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EFFECTS OF POPULATION DENSITY ON THE RATE OF REPRODUCTION IN *OXYTRICHA*

A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF ZOÖLOGY

By
WILLIS HUGH JOHNSON

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EFFECTS OF POPULATION DENSITY ON THE RATE OF REPRODUCTION IN OXYTRICHA[1]

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INTRODUCTION

THE effects of varying the number of Protozoa in a given volume of culture medium and of varying the volume of culture medium for a given number of Protozoa have been investigated by a number of workers in recent years. It is generally recognized that Woodruff (1911*a*) was the first to carefully study this problem. He found a decrease in the rate of reproduction with an increase in the density of the population: "The rate of reproduction of *Paramecium aurelia* and *Paramecium caudatum* is influenced by the volume of the culture medium within the limits tested and the greater the volume the more rapid is the rate of division" (1911*a*, p. 100). He concluded that these results were caused by toxic excretory substances, produced by the organisms themselves, which are more effective in small volumes of culture medium.

This problem remained as Woodruff left it until 1921*a* and 1921*b*, when T. B. Robertson described opposite results with two infusorians, *Enchelys farcimen* and *Colpidium colpoda* (later identified as *Colpoda cucullus*). He found that when two Infusoria (*Enchelys* or *Colpoda*) are introduced into a small amount of fresh culture medium, the early rate of reproduction is more than double that of a single individual of the same species in the same volume of culture medium. This increased rate of reproduction has been designated the "allelocatalytic effect" by Robertson. Allee (1931, p. 166) gives the following statement of Robertson's hypothesis:

> During nuclear division each nucleus retains the charge of autocatalyst with which it was provided, and adds to it during the course of nuclear synthesis. At each division the autocatalyst is shared between the nuclear substance and the surrounding medium in a proportion determined by its relative solubility and by its affinity for chemical substances within the nucleus. The mutually ac-

[1] The writer is greatly indebted to Dr. W. C. Allee for suggesting this investigation and for facilities and advice given in connection with it.

celerative or allelocatalytic effect of contiguous cells is due to each cell's losing less of the autocatalyst to the medium because of the presence of the other. According to this hypothesis, the autocatalytic effect of growing colonies, whether attached or detached, is due to the same cause.

Robertson, in his later papers, stated that this effect is more marked when the organisms have been freed from contamination with the parent culture medium by repeated washings. In his last paper he stated that all of his data and conclusions concerning allelocatalysis in Infusoria were valid except that they might apply to the food organisms. "Whether the allelocatalytic effect originates with the ciliates, or with associated food organisms, however, it remains a mutually accelerative effect of contiguous organisms upon their reproductive rates" (1927, p. 5).

Robertson's results aroused considerable interest; and because of this, several investigators have made similar studies, using different organisms. Cutler and Crump (1923*a* and 1923*b*), using *Colpidium colpoda*, report that they could not obtain the effect described by Robertson. In a like manner Greenleaf (1924, 1926), using *Paramecium aurelia*, *P. caudatum*, *Pleurotricha lanceolata*, and *Stylonychia pustulata;* Calkins (1926), using *Uroleptus mobilis;* Myers (1927), using *Paramecium caudatum*, and Jahn (1929), using *Euglena*, report that they were unable to duplicate the results described by Robertson. On the other hand, Yocum (1927, 1928) reported results similar to those of Robertson, from his work with *Oxytricha*. Peterson (1929) found that she could obtain a decrease or an increase in the relative rate of reproduction of associated and isolated *Paramecium caudatum* by varying the volume of culture medium. When she used $\frac{5}{2^4}$ cc. of medium or less, the two-animal cultures reproduced no faster than a single one in 24 hours. When she used $\frac{20}{2^4}$ cc. of medium, the two-animal cultures reproduced faster than the one-animal cultures. All of the work mentioned in this paragraph has been carefully reviewed by Allee (1931).

Grimwald (1928) found that there was no difference in the division-rate of *Colpidium colpoda*, using one, two, and three organisms in volumes of hay infusion varying from 12.5 to 90 cubic millimeters. In smaller volumes (0.08–12.5) the division-rate was slower than in the larger volumes.

Chejfec (1929) studied the bacterial requirements of *Paramecium caudatum* for the maintenance of life. He states that in a concentration of 24,000 *Bacillus coli* per cubic millimeter a single animal has a normal division-rate. Using concentrations of bacteria much less than the amount needed to give a normal division-rate in *Paramecium*, he reports no evidence of Robertson's effect. He did not investigate the effect of greater concentrations of bacteria on the rate of reproduction of *Paramecium*, using one and two organisms in the same volume of medium.

Darby (1929, 1930) found that *Paramecium caudatum*, *P. aurelia*, and *Stylonychia pustulata* each have an optimum pH for growth and that each organism tends to buffer or bring its medium to its optimum pH. He suggests (1930, p. 312) that

> a possible explanation of Robertson's results appears to be that his medium was away from the optimum pH, and very weakly buffered. The organisms tended to change it, and the more he put in at a time, the quicker the improvement. In the smaller volumes it was easier to bring this about than in the larger ones. In too small a volume, however, the accumulation of ammonia would soon retard the growth; while in the very large volumes the buffering power of the organisms would be ineffective.

In all of the investigations referred to above, only those of Cutler and Crump and Chejfec describe attempts to control the kinds and numbers of bacteria in the culture medium used. The importance of controlling the food supply in such investigations has long been recognized. Jennings (1908) insisted that the bacteria in the culture medium must be the same in any series of experiments. He made no attempt to use pure cultures of bacteria. He took *Paramecia* from vigorous stock cultures, washed them in large amounts of culture medium, and used this washing fluid to seed the medium used for the growth of pure lines of *Paramecium*. He states that, by doing this every few days, he was able to maintain the bacterial content of his cultures at an optimal efficiency as to food.

Hargitt and Fray (1917) plated out bacteria from their protozoan cultures and used pure lines of bacteria in their experimental cultures. Eight of these used singly as food for *Paramecium* were unsatisfactory. *Bacillus subtilis* used alone as food for *Paramecium* proved to be quite satisfactory. Yet they concluded that cultures

of mixed bacteria are, as a rule, better for the growth of *Paramecium* than any single kind of bacteria. They suggest that by using mixtures of known bacteria in sterile infusions better results can be obtained than by using the ordinary mixed cultures of unknown bacteria.

Phillips (1922) found that *Paramecium aurelia* grew better on her A′ and C′ strains of bacteria than on C′ alone. They would not grow on A′ alone or on nine other kinds of bacteria or any combinations of them.

Philpott (1928) reports that *Paramecium* will grow on hay infusions containing only a single kind of pathogenic bacteria, *Bacillus pyocyaneus*. He was not able to obtain growth of *Paramecium* using *Bacillus enteritidis*.

Luck, Sheets, and Thomas (1931) studied the rôle of bacteria in the nutrition of *Euplotes taylori*. Using *Bacillus coli* and five other strains of bacteria alone, they found no favorable growth. When they used two strains of bacteria in the culture, good growth occurred. They later reported that good growth was obtained by using a pure culture of *B. fluorescens pseudomonas*. Barker and Taylor (1931) found that suspensions of a pure culture of the bacterium *Pseudomonas fluorescens* in a balanced physiological medium served as a very suitable food for the ciliate, *Colpoda cucullus*.

Losina-Losinsky (1931) studied the feeding reactions of *Paramecium caudatum*. The results show that bacteria form the chief food substance of *Paramecium*, and that some bacteria are taken in in greater numbers than others. *Paramecium* was found to react quite positively to suspensions of *Bacillus subtilis*, less positively to suspensions of *B. fluorescens liquefaciens*, and very slightly to suspensions of *B. coli communae*.

Stuart and Banta (1931) report that they have been able to grow the cladoceran, *Moina macrocopa*, successfully on suspensions of *Aërobacter aërogenes* introduced into their normal dilute manure infusions. In their work they found that the sex of the young produced by experimentally crowded *Moina macrocopa* mothers can be controlled by the amount of available bacteria present in the medium. In cultures with large numbers of bacteria, the mothers produced only female young. As the number of bacteria was decreased, males

were produced in direct proportion to the decrease in the bacterial count of the medium. From the standpoint of this investigation their work is especially interesting because they found an optimal density of bacteria for maximal reproduction. The greatest number of young was produced, not in the bottles containing the greatest number of bacteria, but in the bottles containing fewer bacteria wherein the sex ratio was approximately three females to one male. At the same time Stuart, McPherson, and Cooper (1931) developed a technique for sterilizing *Moina*. In this part of the work they found that *Moina* requires living bacteria as food, and that all species of bacteria do not serve equally well as food for Cladocera. Some of the bacteria used were actually toxic to *Moina*.

The purpose of the present investigation has been to study the effects of population density on the rate of reproduction in *Oxytricha*, using (1) the common hay infusion with unknown mixed bacteria and (2) a non-nutritive balanced physiological medium in which the kinds and number of bacteria can be controlled.

MATERIALS AND METHODS

Oxytricha fallax was used in all of the experiments. Stock cultures were kept in 200 cc. bottles. Hay infusions were used for the stock cultures. These were prepared, in the initial experiments, by boiling 5 grams of timothy hay in a liter of tap water for 10 minutes. An infusion made with 2 grams of hay proved to be quite suitable and was used for stock cultures in the second phase of the work. The infusion was filtered, when cool, and inoculated with 1 cc. of the preceding culture. Several new stock cultures were started each week, so that a number of flourishing cultures were always on hand. Infusions for the isolation cultures were prepared in the same way, and 200 cc. of the fresh infusion were inoculated with bacteria from a flourishing stock culture of *Oxytricha* 6 hours prior to use. For this purpose 1 cc. of the stock culture fluid, free from *Oxytricha*, was added to the 200 cc. of fresh infusion.

In all of the experiments ground-glass protozoan isolation dishes were used as containers. Evaporation in these small cultures was maintained at a minimum by keeping the dishes in moist chambers. The experiments reported in Tables II–VII were run at room tem-

peratures. All of the other experiments were carried out in a constant temperature room at 27° C., except as noted. In this room the humidity was also high, as a further prevention of evaporation.

Experimental animals were obtained from pure-line isolation cultures. New pure-line cultures were started from stock cultures every 2 weeks. Pure-line cultures containing 32 sister-organisms were used in all experiments. Thus, the organisms used in all paired experiments came from the same isolation culture which started as a single individual. Care was taken not to use either the very small animals or the very large ones ready for division.

The organisms were isolated by means of drawn-out pipettes and were washed twice in 5 drops of fresh medium before they were transferred to the experimental dish. In all of the isolation experiments where hay infusion was used, 24 drops were equal to 1 cc. In the later experiments 18 drops (as reported in Tables X, XII, XIII, XIV, and XV) and 16 drops (as reported in Table XI) were equal to 1 cc.

The hydrogen-ion concentration of the fresh infusions varied from pH 6.6 to pH 7.2, with the greater number of the infusions having an initial pH of 6.8. The hydrogen-ion concentration was determined by the colorimetric method. In those cases where the hydrogen-ion concentration of small cultures was determined at the end of the experiment, the determination was made by a comparison of the unknown with commercial standards in vials of about $\frac{1}{2}$ cc. capacity.

In the second phase of the work, suspensions of bacteria were used in non-nutritive balanced physiological medium. These suspensions were made by removing the bacteria from peptone-glucose-agar plates with a sterile platinum loop and introducing the bacteria into the sterile physiological medium. This medium, originally used by Osterhout (1906) and recently used by Barker and Taylor[2] (1931) in their study of *Colpoda*, is given in Table I.

This solution was diluted two hundred twenty-five times to the approximate concentration of pond water, or 0.012 per cent total salt. One cc. of M/20 NaH_2PO_4 was added to each 30 cc. of this

[2] The author wishes to express his appreciation of the help and advice given by Dr. C. V. Taylor and Mr. H. A. Barker in the development of this technique and in the use of it.

diluted solution for buffering, and the hydrogen-ion concentration was adjusted with small amounts of M/20 NaOH.

In the cases where suspensions of bacteria were used in sterile media, the *Oxytricha* were sterilized by numerous washings in sterile medium, as described by Hargitt and Fray (1917) and Parpart (1928), before they were introduced into the experimental dishes. All dishes and pipettes used in these experiments were sterilized each time before used.

TABLE I

BALANCED PHYSIOLOGICAL MEDIUM

(2.7 per cent total salt [0.375M])

0.375M	Parts
NaCl	1.000
$MgCl_2 \cdot 6\ H_2O$	78
$MgSO_4 \cdot 7\ H_2O$	38
KCl	22
$CaCl_2$	10

Robertson (1924*c*) emphasizes that in experimental work of this kind care must be taken to estimate the population sometime before it has reached the maximal density. In the preliminary experiments in this work, counts were made every 6 hours. On the basis of these results, it seemed desirable to take the counts at the end of 24-hour periods for comparison.

EXPERIMENTS USING HAY MEDIUM

The first set of experiments was run using *Oxytricha* isolated singly and in two's into 1 drop of bacterized hay infusion. Table II shows typical results. At the end of 24 hours there was no difference in the rate of reproduction. However, at the end of 42 hours the fission-rate had slowed down in the two-animal cultures. In no case, in the two-animal cultures, was the total number of animals double that of the one-animal cultures at the end of 42 hours. This seems to indicate that the maximal density had been reached in these cultures sometime between 24 and 42 hours.

A similar set of experiments, using 2 drops of bacterized hay infusion, was carried out. Table III gives the results of a typical series. In this set, likewise, there was no difference at the end of 24

hours between the cultures started with one and with two animals. In half of the cases there were a few more organisms in the original one-animal cultures, and the opposite of this in the other half.

Petersen (1929) reported the same kind of results for her experiments with *Paramecium*, using small volumes of medium. As she

TABLE II

SHOWING THE RESULTS OF 1-DROP EXPERIMENTS

	24-HOUR COUNT		42-HOUR COUNT	
	1-Animal Seeding	2-Animal Seeding	1-Animal Seeding	2-Animal Seeding
Exp. A	4	8	16	25
Exp. B	7	13	31	34
Exp. C	8	16	26	33
Exp. D	7	16	31	41
Exp. E	8	16	18	35

TABLE III

SHOWING THE RESULTS OF 2-DROP EXPERIMENTS

	24-HOUR-COUNT	
	1-Animal Seeding	2-Animal Seeding
Exp. A	8	18
Exp. B	4	9
Exp. C	8	15
Exp. D	7	13
Exp. E	11	24
Exp. F	7	12

had been able to obtain different results using larger volumes, it was deemed necessary to carry out several sets of experiments using larger volumes. *Oxytricha*, isolated singly and in two's into 5 drops of bacterized hay infusion, were used in the experiments reported in Table IV. There were fourteen series in this set of experiments. The number given after each series in this and later tables is the average number of animals produced from a single organism in 24 hours. In each series four dishes with two animals in each and eight dishes with one animal in each were used. Thus, the same number of animals was used in each condition; half of them were isolated

singly, and half in two's. This arrangement was not used in the experiments reported in Tables II and III, but was used in all of the other experiments, as it offered a better basis for comparison. Each series was carried out on a different day. The conditions of the experiments were kept the same except as to temperature. The whole set of experiments was run during the very hot month of August, 1930. The temperature in the laboratory (Whitman Laboratory) reached 34°–35° C. almost every day. The very high rate of repro-

TABLE IV

RESULTS OF 5-DROP EXPERIMENTS

	1-Animal Seeding	2-Animal Seeding
Series 1	17.6	22 5
Series 2	19.1	21.5
Series 3	14.1	16.9
Series 4	30.7	36.4
Series 5	43.0	48.7
Series 6	40.1	37.2
Series 7	23.6	25.1
Series 8	33.9	45.6
Series 9	41.8	44.8
Series 10	18.1	17.7
Series 11	18.6	24.8
Series 12	30.4	39.4
Series 13	18.9	20.1
Series 14	6.9	7.8

duction reported in these experiments must be associated with the very high temperatures. The experiments in Series 14, carried out in much lower temperatures, show a marked decrease in the rate of reproduction. Other experiments, to be referred to later, show the effects of temperature on the rate of reproduction.

An examination of the data in Table IV shows that the average rate of reproduction of the doubles was greater than that of the singles in twelve of the fourteen series. "Student's" method (Student, 1925; Fisher, 1925) of statistical analysis may be applied to such paired experiments to determine their possible statistical significance. Such analysis shows that with the results given in Table IV there are 32 chances in 10,000 of random sampling giving such a wide deviation from the mean. This is well within the range of statistical significance.

Ten drops of bacterized hay medium were used as cultures for a similar set of experiments. The results are given in Table V. The temperature range given is that recorded from a thermometer in the laboratory. These readings show only the temperature range during the day. No attempt was made to obtain the temperatures at night. The effect of temperature on the reproduction-rate is again clearly shown. The data in Table V show that in nine of the fourteen series there was a higher average rate of reproduction with an initial seeding of two as compared with cultures started by a single individual.

TABLE V

RESULTS OF 10-DROP EXPERIMENTS

	1-Animal Seeding	2-Animal Seeding	Temperature Range in Degrees Centigrade
Series 1	21.2	23.5	27–35
Series 2	14.9	16.2	28–33
Series 3	11.6	11.4	26–33
Series 4	9.4	12.9	28–31
Series 5	10.6	12.4	25–30
Series 6	13.2	13.2	27–30
Series 7	19.2	22.4	30–35
Series 8	7.7	7.5	25–30
Series 9	6.9	9.5	25–29
Series 10	7.0	6.6	23–29
Series 11	6.6	6.5	23–28
Series 12	6.6	6.8	23–27
Series 13	7.2	7.4	24–28
Series 14	6.2	6.5	23–26

In one series the average rate was the same, and in the other four the one-animal experiments had a slightly higher average rate. A statistical analysis of these data shows that there are 15 chances in 1,000 of random sampling giving such a wide deviation from the mean. This would generally be taken as significant.

A set of experiments using single animals in 1, 2, 5, 10, and 20 drops of hay medium was conducted. Seven series of these experiments were carried out, using four cultures for each volume mentioned. The averages for all of these gave 33, 32, 28, 23, and 22 as the average number of animals respectively in the 1-, 2-, 5-, 10-, and 20-drop cultures at the end of 24 hours. There was not a significant difference in the 1- and 2-drop cultures. The highest rate of repro-

duction was found in the 1- and 2-drop cultures, with a progressively decreasing rate in the other volumes.

This set-up was repeated using 2-, 5-, 10-, 20-, and 40-drop cultures. Fourteen series were run, using four cultures of each volume in each series. Table VI gives the average results from these experiments. The results of this set of experiments were, in general, the same as those of the preceding set. The highest rate of reproduction

TABLE VI

RESULTS AFTER 24 HOURS OF ISOLATING SINGLE ANIMALS INTO 2-, 5-, 10-, 20-, AND 40-DROP CULTURES

	No. of Drops				
	2	5	10	20	40
Series 1	10.5	9.5	6.0	6.0	5.0
Series 2	7.8	6.2	5.5	4.8	4.5
Series 3	4.8	4.8	4.2	4.0	3.0
Series 4	8.0	7.5	6.5	4.2	3.5
Series 5	6.2	6.7	5.7	4.0	3.5
Series 6	5.7	4.5	4.0	3.2	2.8
Series 7	8.0	7.0	7.0	6.5	6.0
Series 8	6.5	5.2	5.2	5.0	3.5
Series 9	5.0	5.0	4.0	3.5	2.8
Series 10	6.5	5.2	5.2	4.0	3.5
Series 11	5.8	4.5	4.0	4.0	3.5
Series 12	3.5	3.5	3.0	3.0	2.5
Series 13	3.0	2.5	2.0	2.0	1.8
Series 14	8.0	6.5	5.0	3.2	3.0
Mean	6.3	5.6	4.8	4.1	3.5

is found in the 2-drop cultures, with a general decrease with each increase in the volume of the medium. When "Student's" method is applied to the data in Table VI, it is found that there is a significant difference in the average reproduction-rate with each increase in the volume of the medium. That is, the average rate of the 2-drop cultures is higher, and significantly higher, than that of the 5-drop cultures; the average rate of the 5-drop cultures is significantly higher than that of the 10-drop cultures; and so on.

All of the experiments described above were carried out at room temperatures. At this point in the experimentation a constant temperature room with controlled high humidity was made available, and all of the remaining experiments were made with the tempera-

ture controlled. The temperature was kept at 27° C. (unless noted otherwise), and at no time was the variation from this more than 1°. As a check on the previous experiments, a set was carried out, using single animals in volumes of 2 and 20 drops, with the temperature constant. In this set ten cultures for each volume were used in each series. Table VII shows the average results. The maintenance of a constant temperature did not change the type of results obtained. In this set of experiments it is obvious that rate of reproduction in

TABLE VII

RESULTS AFTER 24 HOURS OF SEEDING SINGLE ANIMALS INTO 2- AND 20-DROP CULTURES UNDER CONSTANT TEMPERATURE AT 27° C.

	No. of Drops	
	2	20
Series 1	7.0	5.1
Series 2	3.6	2.9
Series 3	4.2	3.1
Series 4	5.7	3.7
Series 5	3.1	1.8
Series 6	3.1	1.8
Series 7	6.0	3.3
Mean	4.7	3.1

the 2-drop cultures was, in each series, higher than that in the 20-drop cultures.

Maupas (1888), Middleton (1918), Jollos (1921), Dawson (1928), and others have studied rather thoroughly the effects of temperature on the fission rate of various ciliates. They have found that raising the temperature generally causes an increase in the reproduction-rate. This effect, while aside from the main problem, has been noticed in these experiments. This suggested that it would be interesting to compare the results of two sets of experiments like the one given in Table VII, running one set at 70° F. and the other at 80° F., with the temperature constant. Accordingly, such an experiment was made. A comparison showed the following: (1) that the average reproduction-rate of the 2-drop cultures was highest in both sets, (2) that the average reproduction-rate of the 2-drop cultures at 80° F.

was higher than that of the 2-drop cultures at 70° F., and (3) that the average rate of reproduction in the 20-drop cultures at 80° F. was, in most cases, higher than that of the 20-drop cultures at 70° F. This completes the demonstration that the difference obtained in the reproduction-rate in previous experiments is independent of temperature.

Darby (1930) states that he obtained the best results in his work when he used culture dishes coated with paraffin. He suggests that small amounts of toxic materials may leach out of the glass and affect the organisms. It seemed desirable to see if the failure to use paraffin-lined dishes was the cause of the differences in reproduction-rate

TABLE VIII

SHOWING THE EFFECTS UPON THE RATE OF REPRODUCTION OF ANIMALS ISOLATED INTO PARAFFIN-LINED DISHES

	PLAIN		PARAFFIN-LINED	
	2 Drops	20 Drops	2 Drops	20 Drops
Average.....	7.1	5.4	8.4	6.4

already found and described above, as no paraffin-lined dishes had been used in any of the experiments up to this point. Two parallel sets of tests were run—one in plain dishes and the other in paraffin-lined dishes—each seeded with single animals placed in 2- and 20-drop cultures. There were nine parallel series in each set with four cultures for each volume in each series. The average numbers of animals at the end of 24 hours were averaged to give the figures reported in Table VIII.

These results show a higher average rate of reproduction in the paraffin-lined dishes. However, the difference is of doubtful significance. When the 2-drop cultures of the plain and paraffin-lined dishes are compared, there are 17 chances in 100 of random sampling giving such a wide deviation from the mean. When the 20-drop cultures of the plain and paraffin-lined dishes are compared, there are 29 chances in 100 of random sampling giving such a wide deviation from the mean. But the important thing, from the standpoint of this investigation, is that the 2-drop cultures in both the plain and paraf-

fin-lined dishes showed a significantly higher rate of reproduction. As it is difficult to keep the paraffin-lined dishes clean and in good condition, and as their use does not seem to affect the type of results already obtained, it was not considered necessary to use them in all of the future experiments.

Darby (1930), as was mentioned earlier in this paper, found that each of the three ciliates which he studied had an optimum pH for reproduction. Also, he suggests that the type of results found by Robertson might be due to the use of a medium with a hydrogen-ion concentration quite removed from that which is the optimum for reproduction in the infusorian used; under these conditions an infusorian, which he says tends to bring its medium to its optimum pH, placed in the smaller of two volumes, would change the hydrogen-ion concentration faster than would one placed in a larger volume, and thus the difference in reproduction-rate might be accounted for. In view of Darby's suggestion it seemed desirable to study the reproduction-rate of *Oxytricha* in cultures with different hydrogen-ion concentrations. In this work, Darby's method of adjusting the pH of the hay medium was used. This was accomplished by adding 1 cc. of M/20 NaH_2PO_4 to 30 cc. of the hay medium for buffering, and adjusting the pH with suitable quantities of M/20 NaOH. A set of experiments was run, using solutions with initial pH's of 6.0, 6.4, 6.8, 7.2, 7.6, and 8.0. As a control unbuffered hay medium, with a pH of 6.8, was used. In these experiments single animals were isolated into 5 drops of the medium. Eight series were run with four cultures for each pH range in each series. The pH of each culture was measured at the end of 24 hours, when the count was made. Thus, Table IX gives the pH range for the different cultures. Paraffin-lined dishes were used in these experiments.

Each number in Table IX represents the total number of organisms produced from four animals in 24 hours. These data show that the highest rate of reproduction occurred in the control cultures and in the cultures with an initial pH of 6.4. However, the differences are not consistent, and it cannot be stated that there is a very definite optimum indicated in the range used. There is much evidence to show that, while some protozoans have a very narrow pH tolerance, others have a very wide pH tolerance (Wang, 1928). It is indicated

in these experiments that *Oxytricha* belongs to the latter group. Darby (1930) found that, while there was a very marked optimum pH for *Paramecium aurelia* and *P. caudatum*, this was not true for *Stylonychia pustulata*. He suggests that the hypotrichs have a wide pH tolerance.

A sufficient number of experiments were carried out, using single animals in 2- and 20-drop cultures with the hydrogen-ion concentration varied from pH 6.0 to 8.0, to indicate that a difference in the pH of cultures, within these limits, would not change the type of re-

TABLE IX

RESULTS OF VARYING THE HYDROGEN-ION CONCENTRATION WITH SINGLE ANIMALS ISOLATED INTO 5 DROPS OF MEDIUM

	pH RANGE						
	6.0–7.2	6.4–7.4	6.8–7.6	7.2–7.8	7.6–8.0	8.0–8.4	Control 6.8–7.8
Series 1	24	27	17	20	12	14	20
Series 2	32	31	30	32	26	38	28
Series 3	22	26	20	14	14	16	18
Series 4	20	28	20	20	14	21	25
Series 5	22	21	18	16	11	16	20
Series 6	29	29	32	32	25	27	30
Series 7	29	32	30	32	29	20	47
Series 8	14	24	24	20	18	14	30
Av.	24	27	24	23	19	21	27

sults already obtained. Within this range, the rate of reproduction in the 2-drop cultures was always higher than that in the 20-drop cultures.

With a difference in division rate in different population densities clearly indicated by the experiments already reported, and with no obvious cause for the difference, except the volume relations of the animals and culture medium, it seemed desirable to study the food of *Oxytricha* and to find out if the food organisms have any part in causing the difference in reproduction-rate in different population densities.

As the first step in this study, several agar plates were inoculated with some of the liquid from a thriving culture of *Oxytricha*. At the

end of 48 hours each of these plates contained numerous colonies of different kinds of bacteria. Pure cultures of the bacteria from seven of the predominating colonies were grown on agar plates. The ability of *Oxytricha* to grow and reproduce in cultures with a single kind of bacteria as the source of food was tested. Only the predominating kinds of bacteria were used in these tests, because it was considered that the bacteria serving as food for Infusoria in a thriving culture would be fairly abundant in the culture medium. The seven kinds of bacteria isolated in pure cultures were designated as "G," "R," "T," "W," "Y," "S," and "B." "B" was identified as *Bacillus subtilis*. Mr. Barker, who was working in the laboratory at the time, suggested the use of *Pseudomonas fluorescens* which he had in pure cultures. This was done, making eight kinds of bacteria available for use in the food tests.

To determine the ability of *Oxytricha* to grow and multiply, with a single kind of bacteria as the food, necessitated the sterilization of the *Oxytricha* used. This was accomplished by the method of washing suggested first by Hargitt and Fray (1917), with the improvement suggested by Parpart (1928). Medium sterilized in an autoclave was used for the washing fluid. Transfers were made in individual sterilized pipettes. At the end of the twelfth washing, the animals were ready for the test. Five cc. of sterile hay medium in a small sterilized test tube were inoculated with a small amount of a pure culture of bacteria; and into this, five *Oxytricha*, sterilized by twelve washings, were introduced. Three such tests were made for each of the eight kinds of bacteria. The results were the same each time the tests were made. Each time at the end of a week there was a good growth in the cultures inoculated with *Bacillus subtilis* and with *Pseudomonas fluorescens*. In the cultures inoculated with the other types of bacteria the *Oxytricha* were dead at the end of 7 days, with the exception of those cultures inoculated with the bacteria designated as "R." The organisms in these cultures had divided a few times by the end of 14 days, but it was obvious that they were not in a favorable environment. These tests show two things: that at least two kinds of bacteria used alone serve as suitable food for *Oxytricha*, and that at least six other kinds used alone do not.

EXPERIMENTS WITH A NON-NUTRITIVE MEDIUM

In an analysis of the effect of bacteria on the rate of reproduction in *Oxytricha*, it seemed necessary to control the numbers of bacteria. This is not possible in the use of a nutritive hay medium. At the suggestion of Dr. C. V. Taylor, the use of a balanced physiological medium (given in Table I) diluted to the approximate concentration of pond water was used. In the beginning it was necessary to find out what concentration of bacteria proved most suitable for reproduction in *Oxytricha*. A number of different-sized platinum loops were made and a loopfull of bacteria was introduced into 10 cc. of the balanced medium. Suspensions of bacteria so made were used for culturing the *Oxytricha*, and the concentration giving the most satisfactory results was used for further experimentation. Such a test was made for both *Bacillus subtilis* and *Pseudomonas fluorescens*.

With the optimal density of bacteria (for the two kinds just mentioned) for favorable reproduction determined, a set of parallel experiments was run, using single *Oxytricha* introduced into a suspension of *Bacillus subtilis*, a suspension of *Pseudomonas fluorescens*, an equal mixture of the two bacterial suspensions, and into the ordinary hay infusion. Two drops were used in each case. The pH of the balanced medium was 6.8 at the beginning of the experiments. The pH was adjusted as outlined in the section on methods. This pH was used because it was the same as that of most of the hay infusions in the previous experiments and because the studies reported in Table IX showed this pH to be well within the range for good reproduction. In this, and in all other sets of experiments to be reported, the temperature was held at 27° C. Table X shows the results of these experiments.

Each number in Table X is the average number of animals produced in four dishes at the end of 24 hours, when each dish started with a single animal. These experiments show that there was a distinctly higher rate of reproduction in the suspensions of *Pseudomonas fluorescens* than in any other type of culture. Suspensions of *Bacillus subtilis* proved to be the least suitable. As might be expected, a mixture of the two was more suitable than a suspension of *B. subtilis* alone. In no case was the rate of reproduction in the hay infusion cultures nearly as high as that in the suspensions of *Pseudomonas*

fluorescens. It averaged about the same as the average rate in the mixed suspensions. This work clearly shows that suspensions of *Pseudomonas* are more suitable than ordinary hay infusions for this type of experimentation with *Oxytricha;* accordingly, suspensions of this kind of bacteria were used in the further analysis of the problem.

It seemed logical to suppose that bacteria introduced into the non-nutritive physiological medium would not increase in numbers. However, a check on this supposition was made. A standard suspension

TABLE X

SHOWING THE REPRODUCTION-RATE FOR 24 HOURS OF *Oxytricha* INTRODUCED SINGLY INTO THE BALANCED PHYSIOLOGICAL MEDIUM AND INTO THE HAY MEDIUM

	KIND OF MEDIUM			
	Suspension of *Pseudomonas fluorescens*	Suspension of *Bacillus subtilis*	Mixed Suspension	Hay Infusion
Series 1	8	2.2	3	5
Series 2	18.7	9.2	9	12
Series 3	16	5.2	11.5	9.2
Series 4	27.7	10.2	16.5	15.5
Series 5	21	11.2	13.7	10.7
Series 6	18.5	7.5	11	15
Series 7	11.5	4.5	6	?
Series 8	14	12	10	11
Series 9	28.7	13	18.8	18.5
Av.	18.2	8.3	11.1	11.6

was made by introducing a loopfull of bacteria into 10 cc. of sterile balanced medium in a test tube. This standard suspension was the concentration which had been found to be most suitable for reproduction in *Oxytricha*. One cc. of this standard suspension was diluted 200,000 times with sterile balanced medium. One drop of this suspension (16 drops equal 1 cc.) was spread on an agar plate, and the colonies of bacteria formed after 48 hours were counted. The standard suspension was kept for 24 hours, and after the suspension was mixed thoroughly this process was repeated. By comparing the number of colonies formed at the beginning and at the end of 24 hours, one could determine whether the bacteria had increased in

numbers. Table XI gives the results of a number of these comparisons.

In this work the bacteria cultures were from 5 to 12 days old. In the first six series in Table XI each number is the average number of colonies found on four plates. In Series 7 each number is the average number of cultures found on twelve plates. All of the plates in each series were made from the same standard suspension. The data in Table XI show that there is some decrease in the number of bacteria in the balanced medium during 24-hour periods. On such evidence it was assumed that there would be little, if any, reproduction of the bacteria in similar suspensions containing the *Oxytricha*.

TABLE XI

SHOWING THAT THE BACTERIA SUSPENDED IN THE BALANCED MEDIUM DO NOT INCREASE IN 24 HOURS

	No. at the Start	No. at the End of 24 Hours
Series 1	37	30
Series 2	25	21
Series 3	34	28
Series 4	37	35
Series 5	40	32
Series 6	35	28
Series 7	31	27

If the numbers in Table XI, which represent average numbers of colonies formed from individual bacteria, are multiplied by 200,000 $\times$16, or 3,200,000, the average number of bacteria in 1 cc. of the standard suspension will be obtained. This number ranges from 80 to 128 million per cubic centimeter for the experiments reported in Table XI. During the course of the remaining experiments, nine series of bacterial counts similar to those just mentioned were made at different times. The average number of bacteria in millions per cubic centimeter of standard suspension were: 135, 100, 98, 118, 128, 133, 129, 113, 90, and 111. This range is practically the same as that found in Table XI. The variation is undoubtedly due in part to the method of measuring the bacteria in a platinum loop, although the same loop was used throughout and a sterile scalpel was used to remove all bacteria not actually in the loop. At any rate, with these

counts as a basis, an approximation of the number of bacteria in any culture can be made. In the remaining experiments, "X" is used to designate the standard suspension of bacteria. Dilutions of this standard suspension are indicated by fractions, i.e., X/4 and X/10. Suspensions of greater density are indicated by 2X, 4X, and 5X. The dilutions were made by diluting the standard suspension by the amount indicated by the fraction. The suspensions of greater density were made by adding two, four, or five times the standard amount of bacteria to 10 cc. of the balanced medium.

TABLE XII

RESULTS OF THE ISOLATION OF SINGLE ANIMALS INTO 2 DROPS OF DIFFERENT CONCENTRATIONS OF BACTERIA

	CONCENTRATION				
	4X	2X	X	X/4	X/10
Series 1	4.75	8.75	7.50	4.75	2.25
Series 2	4.00	12.25	14.50	5.00	2.25
Series 3	3.50	14.00	15.25	5.00	3.50
Series 4	2.50	8.75	10.00	3.50	2.25
Series 5	5.50	8.00	8.50	5.75	3.00
Series 6	4.00	11.50	15.75	4.00	0.50*
Series 7	2.00	5.50	8.00	6.50	2.00
Series 8	3.75	7.00	8.00	5.00	3.75
Series 9	3.50	9.50	15.25	5.50	4.25
Series 10	1.50	5.00	9.25	7.00	3.25
Series 11	1.50	6.00	10.75	7.00	4.75
Series 12	6.00	11.75	14.00	5.50	4.00
Mean	3.54	9.00	11.38	5.37	2.98

* Animals in some of the cultures died.

A set of experiments was carried out using single animals isolated into 2 drops of bacterial suspensions of 4X, 2X, X, X/4, and X/10 concentrations. The counts were made, as usual, at the end of 24 hours; the results are given in Table XII. The animals used in this and in the remaining sets of experiments were taken from pure-line cultures grown on the standard suspension of bacteria. Each number in this table is the average number of animals produced from four cultures, each starting with a single individual.

These results bring out the fact, already referred to, that there is an optimal concentration of *Pseudomonas* for reproduction in *Oxy-*

tricha. An analysis of these results shows that there is a significant difference in rate of reproduction with each increase or decrease in the concentration of bacteria from the standard suspension. The decrease in reproduction-rate in the dilute suspensions is probably due to a lack of the optimal amount of food. The cause for the decrease in the more concentrated suspensions is not so obvious. This will be taken up in the discussion.

TABLE XIII

REPRODUCTION-RATE FOR 24 HOURS FOR ONE AND TWO ANIMALS IN 2 DROPS OF 4X, 2X, AND X CONCENTRATIONS OF *Pseudomonas fluorescens*

	Concentration					
	4X		2X		X	
	1-Animal Seeding	2-Animal Seeding	1-Animal Seeding	2-Animal Seeding	1-Animal Seeding	2-Animal Seeding
Series 1.....	5.25	6.50	8.75	14.37	7.50	7.25
Series 2.....	2.75	4.50	12.25	13.00	14.50	14.00
Series 3.....	6.00	7.12	14.00	15.37	15.25	16.37
Series 4.....	6.75	7.12	8.75	10.25	9.50	8.87
Series 5.....	7.25	8.75	8.00	8.37	10.50	10.87
Series 6.....	2.75	4.12	10.50	13.75	15.75	14.75
Series 7.....	3.75	4.87	5.00	7.62	10.00	10.75
Series 8.....	3.50	4.00	7.00	8.62	8.50	9 00
Series 9.....	1.25	2.00	4.00	5.50	9.25	8.00
Series 10.....	1.50	2.12	4.00	5.12	10.75	8.37
Series 11.....	4.25	5.50	8.00	8.50	8.00	7.87
Series 12.....	5.50	5.75	8.00	11.62	8.00	8.12
Mean...	4.21	5.19	8.02	10.17	10.62	10.35

With an optimal density of bacteria for reproduction of *Oxytricha* clearly indicated, two sets of experiments were run in which animals were introduced singly and in two's in different concentrations of bacteria. The results of the use of 4X, 2X, and X concentrations are listed in Table XIII. In each series two dishes with two animals in each and four dishes with one animal in each were used for each concentration of bacteria. The numbers listed are the averages of either 2 two-animal dishes or 4 one-animal dishes at the end of 24 hours.

The results given in Table XIII show that there was a higher rate of reproduction in the two-animal cultures in both the 4X and 2X concentrations. These differences are significant. These results are

similar to those obtained in the earlier experiments using hay medium. There was no consistent difference in the rate of reproduction of the one- and two-animal cultures in the X concentration. These results seem to indicate that the density of the bacteria is a major cause of the difference in the fission-rate of one- and two-animal cultures. When densities greater than the optimum are used, it appears that two animals are able to reduce the density of the bacteria

TABLE XIV

REPRODUCTION-RATE FOR 24 HOURS FOR ONE AND FOUR ANIMALS IN 2 DROPS OF 5X AND X CONCENTRATIONS OF *Pseudomonas fluorescens*

	Concentration			
	5X		X	
	1-Animal Seeding	4-Animal Seeding	1-Animal Seeding	4-Animal Seeding
Series 1.....	2.62	3.46	8.00	5.31
Series 2.....	2.25	3.50	9.00	8.81
Series 3.....	2.75	2.62	12.00	10.68
Series 4.....	0.37*	0.93*	7.25	5.87
Series 5.....	1.25	1.53	8.00	8.00
Series 6.....	0.62*	1.50*	6.00	5.00
Mean.....	1.65	2.25	8.38	7.28

* Animals in some of the cultures died.

toward the optimum quicker than one animal, and thus are able to reproduce faster in the initial stages of the culture.

In a similar set of experiments, one- and four-animal cultures in 5X and X concentrations were studied. The results are shown in Table XIV. In each series two dishes with an initial seeding of four animals in each and eight dishes with one animal in each were used for each concentration of bacteria.

The data in Table XIII indicate that there is very little difference in the rate of division in one- and two-animal cultures in X concentrations of bacteria. Apparently, 2 drops of the standard suspension furnish adequate food for the progeny of two *Oxytricha* during 24 hours. When these results are compared with those of the X concentrations given in Table XIV, we see that the four-animal cultures, in all except one series, had a lower average reproduction-rate than the

one-animal cultures. The difference borders on significance. This seems to indicate that there is not sufficient food in X concentrations for the progeny of four *Oxytricha* during 24 hours. An examination of the cultures of 5X concentration shows that little reproduction occurred in any of the cultures. In several of the cultures some of the protozoans died. It seems obvious from this part of the study that in bacterial suspensions of such great density the protozoans are killed. The differences between the reproduction-rates in the one- and four-animal cultures of this concentration are of questionable significance.

TABLE XV

REPRODUCTION-RATE FOR 24 HOURS FOR SINGLE ANIMALS IN 2 AND 10 DROPS OF X AND X/10 CONCENTRATIONS OF *Pseudomonas fluorescens*

	Concentration			
	X		X/10	
	2 Drops	10 Drops	2 Drops	10 Drops
Series 1.....	6.50	7.50	2.00	5.25
Series 2.....	8.00	7.00	3.75	7.00
Series 3.....	8.00	8.50	5.25	7.50
Series 4.....	16.00	14.00	2.25	4.25
Series 5.....	14.00	13.50	3.50	5.00
Series 6.....	8.00	7.50	3.50	7.00
Series 7.....	13.25	12.00	0.50*	3.50
Series 8.....	9.50	9.00	3.00	7.50
Series 9.....	7.50	7.75	0.50*	1.75
Series 10.....	11.00	12.00	5.00	7.50
Mean.....	10.17	9.87	2.92	5.62

* Animals in some of the cultures died.

The last set of experiments to be reported in this paper consisted of a study of the rate of reproduction of single animals isolated into 2 and 10 drops of X and X/10 concentrations. The results given in Table XV afford some interesting comparisons. There is no significant difference in the rate of reproduction in the 2- and 10-drop cultures of X concentration. In the earlier experiments, when the nutritive hay medium was used, there was always a significant difference with this variation in volume. There is a very significant difference in the reproduction-rate of the cultures of X/10 concen-

trations: the 10-drop cultures have the higher rate. The 10-drop cultures contained five times the amount of food present in the 2-drop cultures. As 2 drops of X/10 concentration have already (Table XII) been shown to be inadequate for normal reproduction, the results here are not surprising. There is quite a difference in the results obtained in the 2-drop cultures of X concentration and in the 10-drop cultures of X/10 concentration. When the averages for these cultures are compared, one finds that the reproduction-rate of the 2-drop cultures of X concentration is much higher than that of the 10-drop cultures of X/10 concentration. The 10-drop cultures of X/10 concentration contain only half the amount of bacteria found in the 2-drop cultures of X concentration. Whether this is the only factor operating, or whether a difference in the availability of the bacteria is a factor, cannot be stated at this time.

DISCUSSION

In the first part of this work, when hay medium was used, two kinds of results were obtained, depending upon the volume of the culture medium. The rate of reproduction per animal of two *Oxytricha* seeded together was greater than that of one animal in volumes of 5 and 10 drops of medium. This was not the case in volumes of 1 and 2 drops of medium. This volume relationship was further indicated in the experiments reported in Table VI, where a decrease in rate of reproduction occurred with each increase of volume above 2 drops. These results are essentially like those found by Petersen (1929), who used *Paramecium*. This is the type of result which Robertson (1921*a* and 1921*b*) and later Yocum (1928) referred to as "allelocatalysis." As a number of workers have obtained different results in similar experiments, an attempt has been made here to further analyze the cause for these results.

The experiments reported show that the results are independent of temperature. It is shown that the results are not affected significantly by the use of paraffin-coated culture dishes. Also, variations in the hydrogen-ion concentration of the medium have not altered the type of results obtained. Of the various factors investigated, food alone offers a basis for a possible explanation of the differences discovered in the different volumes of hay medium used.

Robertson (1927) suggested that *Colpidium* and other ciliates are possibly restricted in their food to certain species of bacteria. He further suggested that the allelocatalytic effect might originate with the bacteria but that, regardless of whether it originated with the ciliates or with the bacteria, the results would be the same. In another place he suggested that the differences between his work and that of Cutler and Crump might be due to differences in the protozoan-bacteria ratio due to differences in technique. Other investigators have recognized the need of a plentiful supply of bacteria in the cultures. In most of the investigations, the presence of numerous bacteria in the cultures, introduced with the protozoan or by seeding from another culture, has been considered to be a sufficient control of the bacteria. Cutler and Crump (1924) reported that the bacteria found associated with *Colpidium* served as a better food organism than *Sarcina*, which they used. These investigators studied the effects of different numbers of bacteria on reproduction in *Colpidium* and found that an increase of bacteria caused an increase in the rate of reproduction. They did not, however, study the effects of very large numbers of bacteria, nor did they tie this up with their work on allelocatalysis. Obviously, it is impossible to analyze the results of the different investigators from the standpoint of food, when the kinds of bacteria, the suitability of the bacteria as food for the protozoan used, and the numbers of bacteria in the cultures are not known in most of the cases.

The results in the second phase of this work show that some bacteria used alone serve as suitable food for *Oxytricha*, while other kinds of bacteria used alone do not. These results are similar to those of Hargitt and Fray (1917), Phillips (1922), Philpott (1928), Losina-Losinsky (1931), and Luck, Sheets, and Thomas (1931); they seem to indicate that the presence of bacteria in large numbers in a culture does not necessarily mean that all of the bacteria are suitable food for a particular protozoan. Thus, the need of a rigid control of the kind or kinds of bacteria used in experiments on the reproductive rate in protozoans is desirable.

Not only the kinds of bacteria, but also the numbers of bacteria, are important in this work. The preliminary tests with bacterial suspensions, together with the results given in Tables XII, XIII,

XIV, and XV, show that an optimal density of bacteria is required for a good rate of reproduction in *Oxytricha*. This is not the first report of the dependence of reproduction in protozoans on an optimal density of bacteria. Chejfec (1929) found that the division-rate of *Paramecium* depends on the number of bacteria. He states that if the concentration of bacteria is too great, the division-rate is slow until an optimal number of bacteria is reached by the Infusoria eating them. The accompanying table (XVI), taken from Chejfec, illustrates his findings.

TABLE XVI

INDIVIDUAL NUMBER IN DIFFERENT CONCENTRATIONS OF *Bacillus coli* AFTER 2, 4, AND 6 DAYS (FROM CHEJFEC)

DAYS	INITIAL SEEDING	*Bacillus coli* PER CUBIC MILLIMETER				
		25,000	50,000	75,000	125,000	175,000
2	25	366	323	224	197	127
4	25	583	783	1,665	1,872	2,428
6	25	486	1,045	1,686	3,476	6,680

Barker and Taylor (1931), in using suspensions of *Pseudomonas fluorescens* as food for *Colpoda*, also found that reproduction is dependent upon the density of the bacteria. They say:

> The density of the bacterial suspension provided for the Protozoa is difficult to define except as that density in which optimal growth occurs. If too many bacteria surround the Protozoa, the latter are adversely affected as shown by a strongly reduced division rate. Definite pathologic forms may even appear. In an exceedingly dilute suspension of bacteria the Protozoa show reduction in size and division rate. In short, they behave like any undernourished organisms. Between these two extremes is a relatively wide range of bacterial suspension densities in which the division rate and the size of the *Colpoda* are maximal and approximately constant.

Some of the observations on *Drosophila* suggest a similar relationship. Eigenbrodt (1925) found that *Drosophila* grow larger in small culture vials when present in numbers of from 8 to 16 than at other population densities. Allee (1931) suggests that these observations of Eigenbrodt may be explained on the assumption "that too few *Drosophila* larvae per culture fail to control the growth of harmful

elements of the yeast or bacterial flora as well as optimal numbers do, while overcrowding overcontrols the growth of the food plant."

In the present studies, suspensions of *Pseudomonas fluorescens* in densities of 80 to 135 million per cc. (X concentration) in non-nutritive medium have been found to be optimal for reproduction in *Oxytricha*. Two drops of such suspensions (18 drops in 1 cc.) will support the progeny of either one or two *Oxytricha* for 24 hours without any significant difference in the division-rate. The fission-rate of four *Oxytricha* in the same volume of this concentration is less than that of one. In suspensions of greater density the rate of reproduction decreases with each increase in density. The use of 5X concentrations, as shown in Table XIV, almost inhibits reproduction, and in some cases causes death. Four animals in 2 drops of this suspension are unable to decrease the numbers of bacteria rapidly enough to prevent this effect.

The higher rate of reproduction found in the two-animal cultures in both 2X and 4X concentrations of bacteria is much like the type of results reported by Robertson, Yocum, and Petersen. In these experiments the cause for the results seems to be largely due to the supra-optimal densities of bacteria. As Chejfec (1929) has pointed out, when the concentration of bacteria is too great, the division-rate is slow until an optimal number of bacteria is reached by the Infusoria eating them. Two animals are able to do this faster than one, and thus, in the two-animal cultures in supra-optimal densities of bacteria, the reproduction-rate is higher than that in the one-animal cultures.

When single animals were used in 2 and 10 drops of X and X/10 concentrations, the rate of reproduction was practically the same in the cultures with X concentration of bacteria, but quite different in those of X/10 concentration. The presence of five times the amount of bacteria in 10 drops as compared with 2 drops of X/10 concentration will explain the higher reproduction in the former. As the 10-drop cultures of X/10 concentration contain only half the amount of food contained in the 2-drop cultures of X concentration, the great difference in the results may be due to the difference in the amount of the food in the two cases. However, a difference in the

availability of the bacteria suggests itself as another possible factor. More work is needed in this connection.

Although in the X/10 concentrations the 10-drop cultures had a higher rate of reproduction than the 2-drop cultures, there was almost no difference in the reproduction-rate of the 2- and 10-drop cultures of X concentration. The use of similar volumes of hay medium always gave differences in the reproduction-rate. This suggests that the rate of reproduction of *Oxytricha* for 24 hours is independent of the volume of the medium, within this range, when the density of the bacteria is optimal.

These results with suspensions of bacteria suggest that the results obtained with the hay medium may be due to the effects of the bacteria. It is taken for granted that in the hay cultures sufficient numbers of suitable bacteria were present to permit reproduction of the protozoans. In small volumes of the hay medium (1 and 2 drops) the amount of nutritive material present is much less than that in larger volumes (5, 10, 20, and 40 drops). Because of this, the larger volumes are able to support greater numbers of bacteria. In the first set of experiments it was found that 1 drop of hay medium, seeded with bacteria as described, supports the progeny of either one or two *Oxytricha* for 24 hours without any difference in the rate of reproduction. But at the end of 42 hours the one-animal cultures show a higher rate of reproduction. This seems to be due to a quicker exhaustion of the food supply in the two-animal cultures. In other experiments, when 5- and 10-drop cultures were used, the two-animal cultures had a higher rate of reproduction at the end of 24 hours. These volumes of hay medium will support greater numbers of bacteria than 1 drop will. From the data obtained, when using bacterial suspensions where the numbers of bacteria were known, it seems plausible to suggest that the effect of bacterial crowding was occurring in the larger volumes of hay medium which would account for the progressive decrease in reproduction-rate with progressive increases in volume of medium, and also for the higher reproduction-rate in the two-animal cultures of 5- and 10-drop volumes. The observation that dense clouds of bacteria existed at the end of 24 hours in the 10-, 20-, and 40-drop cultures seems to support this suggestion.

The results given in Table XIII are similar to those reported by

Robertson, Yocum, and Petersen. Those given in Table XV are similar to those reported by Woodruff, Myers, Calkins, Cutler and Crump, Greenleaf, and others. The differences in the results reported here are due to differences in the density of the bacteria in the medium. These findings suggest that the differences in the results obtained by different investigators may be due to differences in the numbers of bacteria in the cultures used. Furthermore, in considering reasons for the differences in results obtained by different investigators, the effects of unsuitable bacteria in hay infusions of unknown bacterial content should not be overlooked.

In this work no attempt has been made to prove or disprove the existence of an "X-substance" as postulated by Robertson. The results obtained here, with the bacteria controlled, show that the density of the bacteria in cultures of *Oxytricha* will explain to a considerable degree, and perhaps completely, the differences obtained in the present series of experiments upon the rate of reproduction in different population densities. Robertson reported that the interfission period was progressively shortened in the early stages of the culture. Petersen states that she did not find this to be the case. This point has not been investigated in the present work. More work is necessary in this connection, as there is some basis, on the results reported here, for supposing that protozoans in supra-optimal densities of bacteria might have their interfission periods shortened as they approach optimal densities of bacteria in the culture.

The use of conditioned medium, or medium in which an organism has lived and divided and then removed, has not been made in these experiments. The effects of toxic excretory substances, a possible "X-substance," and a decrease in the number of bacteria in the culture so that insufficient food is left, have been suggested by different investigators as having a part in the conditioning of the medium. This work indicates that other factors may enter into the conditioning, as (1) the protozoan may reduce supra-optimal numbers of bacteria to the optimum, (2) the introduced protozoan may bring into the culture more suitable bacteria, and (3) the introduced protozoan may bring into the culture harmful bacteria. That excretory substances play an important rôle in cultures of any duration is obvious. The relative rôles of food and excretory substances can be tested by

using the above mentioned technique. By using suspensions of bacteria in a given volume of non-nutritive medium, the bacteria can be replenished without changing the medium. A record of the reproduction-rate over a few days should show when excretory substances become more important than food in controlling the reproduction-rate. Experiments on this point are needed.

Further work is also needed to determine the number of bacteria consumed by a single protozoan and its progeny during the 24-hour period in different densities of bacteria. Such information should shed more light on the part played by different bacterial densities on the rate of reproduction of protozoans.

SUMMARY

1. When *Oxytricha* were isolated singly and in two's into 1 or 2 drops of hay medium, there was no difference in the rate of reproduction during 24 hours.

2. In similar experiments using animals isolated singly and in two's into 5 and 10 drops of hay medium, a higher reproduction-rate was found in the cultures starting with two animals.

3. Hay cultures, varying in volume from 1 to 40 drops and inoculated with single animals, showed the highest rate of reproduction in the 1- and 2-drop cultures and progressively decreasing rates in the 5-, 10-, 20-, and 40-drop cultures.

4. These results were found to be independent of temperature, as far as tested; of variations in the hydrogen-ion concentration of the medium within the range used; and of the use of paraffin-lined culture dishes.

5. Several of the experiments showed that increases in temperature caused increases in the reproduction-rate of the protozoans, according to usual expectations, but that relations mentioned above were not affected.

6. Suspensions of the bacterium *Pseudomonas fluorescens* in a non-nutritive balanced physiological salt medium were found to be the best type of culture medium tried for *Oxytricha*. Suspensions of *Bacillus subtilis* were less helpful, while other pure cultures of bacteria tested did not allow reproduction of *Oxytricha*.

7. Suspensions of *Pseudomonas fluorescens* in densities of 80–135

million per cubic centimeter were found to be optimal for maximal reproduction in *Oxytricha*. There was no difference in the average reproduction-rate of one and two animals for 24 hours in 2 drops of this concentration.

8. When single animals were isolated into 2 drops of medium with bacterial densities of *Pseudomonas fluorescens* two and four times greater than the optimal density, the rate of reproduction was progressively lower with each increase in density. In these supra-optimal densities, two-animal cultures had a higher reproduction-rate for 24 hours than one-animal cultures.

9. Cultures with both one and four animals, in five times the optimal density of *Pseudomonas fluorescens*, showed a marked inhibition of reproduction. In some cases the animals died in this density.

10. The rates of reproduction of single animals in 2 and 10 drops of optimal densities were about the same. The rates of reproduction of single animals in 2 and 10 drops of medium with bacterial concentrations one-tenth that of optimal concentrations were much less than those in optimal concentration, but the 10-drop cultures have the higher rate of reproduction.

11. These results show that many of the so-called allelocatalytic results obtained by Robertson can be explained on the basis of the ratio existing between the infusorian and the bacterial population.

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